Deoxyribonucleic acid of *Cancer pagurus*. II. Template activity for a DNA-dependent DNA polymerase of eukaryotic cells

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ABSTRACT

The template activity of *Cancer pagurus* DNA and its two components (poly d(A-T) and main component) in response to a DNA polymerase purified from regenerating rat liver has been studied and compared to the results previously obtained with synthetic templates. In the double-stranded native state, whole crab DNA and the main component were poor templates. Their replication was increased by thermal denaturation and inhibited by actinomycin. Like the synthetic copolymer poly[d(A-T).d(T-A)], native crab poly d(A-T) could be copied and its duplication was not inhibited by actinomycin. The structural difference between native poly d(A-T) Form I, isolated on a density gradient, and partially renatured poly d(A-T) Form II, isolated on hydroxylapatite, resulted in a modification of their template activity. The kinetic studies of [³H]dGMP and [³H] dAMP incorporation confirmed the importance of single-stranded regions (particularly dC regions) in the initiation of the in vitro duplication.

INTRODUCTION

Crab poly d(A-T) has been used as template for the DNA polymerase I of *E. coli*¹ and for different DNA-dependent RNA polymerases²⁻⁴ to determine the distribution of G-C bases along the molecule of this satellite. Recently, Berger et al.⁵ showed that in the presence of purified liver DNA polymerase, crab poly d(A-T) gave rise to an autocatalytic synthesis until complete exhaustion of the input deoxyribonucleotides had occurred. To our knowledge, no comparative study of the template activities of total DNA, of poly d(A-T) and of the main component has been made.

We had previously purified a DNA polymerase that is very sensitive to the secondary structure of the template and whose specificity with regard to different synthetic templates is known⁶. It appeared interesting to use this enzyme to study the template properties of natural DNAs, whose secondary structure and base composition are different. This DNA-dependent DNA polymerase, purified from regenerating rat liver, requires a partially single-stranded heat-denatured DNA template or a homopolymer initiated by a complementary oligomer. The enzyme has a very strong affinity for single-

stranded poly (dC) ; the alternating copolymer poly[d(A-T).d(T-A)] is the only double-stranded model that it can copy. We therefore studied the template activity of Cancer pagurus DNA and compared it with the activities of the main component and of poly d(A-T) separated according to previously described methods : density gradient, chromatography on hydroxylapatite or on methylated albumin⁷. We also studied the activities of mixed templates in which each constituent (poly d(A-T) and main component) were at known proportions.

MATERIAL AND METHODS

DNA-dependent DNA polymerase of regenerating rat liver : The enzyme was purified 500 to 1000 times according to a method published elsewhere⁶ which includes the following steps : centrifugation at 105.000 g for the supernatant (Fraction I), precipitation with ammonium sulfate (Fraction II), chromatography on DEAE cellulose (Fraction III) and chromatography on hydroxylapatite (Fraction IV). At this step, the enzymatic preparations have a specific activity of 100 to 150 units per mg of protein in the presence of synthetic poly[d(A-T).d(T-A)] and a concentration of 800 µg per ml or less. One unit of DNA polymerase is defined as the activity necessary to convert 1 nmole of nucleotide to an acid-insoluble product in 1 hour under the standard conditions indicated below.

The incubation medium with a final volume of 0.250 ml had the following composition : 60 mM Tris-HCl (pH 7), 2.4 mM KCl, 3.4 mM MgCl₂, 100 mM 2-mercaptoethanol, 200 µM deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP) which contained 2 µCi of a radioactive marker, 3 to 12.5 µg of template (depending on the experiment) and 100 µl of enzyme (Fraction IV). After incubation at 38°C, the acid-insoluble part of an aliquot fraction was precipitated on Whatman GF/C glass filter. The filter was washed with perchloric acid (5% perchloric acid, 2% sodium pyrophosphate), then dried in alcohol and ether. The radioactivity of the product retained on the filter was determined by counting in a PACKARD scintillation counter in the presence of a toluene-dimethyl POPOP-PPO mixture. In our experimental conditions, with 100 µg of enzyme, 10 µg of crab poly d(A-T) are duplicated in three hours.

Nuclease of *Neurospora crassa* : This endonuclease, specific for single-stranded DNA, was prepared according to Linn & Lehman^{8,9} and was given us by Dr. M. Girard.

For the reaction, 0.07 unit of the nuclease was added to 12.5 µg of DNA or poly d(A-T) for 30 minutes at 38°C in the presence of 10 mM Mg⁺⁺,

1 mM 2-mercaptoethanol, 100 mM Tris-HCl (pH 7.5) in an incubation medium of 100 μ l ; the polymerization reaction was then conducted under the previously described conditions after the addition of the deoxynucleoside triphosphates and 100 μ l of DNA-polymerase.

Templates : Total DNA was prepared according to Baranowska et al.¹⁰ and was composed of molecules with a relatively high molecular weight (3 to 7.10^6 daltons, depending on the preparation).

The two components of total DNA were separated either by cesium sulfate gradient centrifugation after Hg-ion fixation on thymidine¹¹ or by chromatography on hydroxylapatite at 70°C¹². Under these conditions, the main component contains 43% of G.C base pairs and the light satellite, representing 20% of the total DNA, contains only 3% of guanine-cytosine. The latter is essentially an alternating copolymer of A and T, but non-alternating dA.dT form about 7% of this satellite with 65% of G.C base pairs in isolated positions¹³.

Davidson's method gives poly d(A-T) in its native form or Form I. The chromatography on hydroxylapatite at 70°C yields poly d(A-T) in its partially renatured form or Form II. In fact, the non-alternating dA.dT are responsible, together with the G.C base pairs, for its incomplete renaturability^{7,13}.

The purity of poly d(A-T) samples (Form I or II) was verified by thermal dissociation curves and analytical ultracentrifugation.

Poly d(A-T) enriched DNAs were prepared by fractionating total native DNA on a MAK column at room temperature according to the method previously described⁷. Under these conditions, the first fractions eluted contained only the main component ; they were pooled and called peak A. Then the poly d(A-T) increased and represented 45% of peak B ; in this case, the poly d(A-T) was in the native form.

The physicochemical properties of the samples, as well as the mixture prepared from poly d(A-T) (Form I or II) and the isolated main component, are described in a previous article⁷.

Substrates : The deoxyribonucleoside triphosphates (dATP, dGTP, dCTP and dTTP) were obtained from SCHWARTZ MANN (Orangeburg New York 10962). The markers used, [³H] dTTP, [³H] dCTP, [³H] dGTP and [³H] dATP, were from the RADIOCHEMICAL CENTER (Amersham, Buckinghamshire, England).

Actinomycin : Actinomycin (MERCK) in a ethanol/water (v/v) solution, was used at doses calculated according to the base composition of the template so that the actinomycin/G ratio was 2.

EXPERIMENTAL RESULTS

Template activity of total DNA and its two components ; influence of thermal denaturation :

Figure 1 shows the rate of synthesis in the presence of the different templates with or without previous heat denaturation. We used [^3H]dATP and [^3H]dGTP to follow the copy of the A-T and G-C sequences respectively.

Like other natural DNAs, total crab DNA is only partially copied in its native state, but thermal denaturation clearly increases both dGMP and dAMP incorporation (Fig. 1a). The same was observed for the the main component, when separated from poly d(A-T) either by chromatography on hydroxylapatite or by density gradient (Fig. 1b). With poly d(A-T) (Fig. 1c and 1d), we observed that replication of G-C sequences was only slightly increased after heat denaturation whether the light satellite was obtained by density gradient (Form I) or by chromatography on hydroxylapatite (Form II). This implies that the G-C sequences are very short and that they cannot resist the unwinding of the double helix by DNA polymerase or that they are partially denatured by the poly d(A-T) isolation procedures. However, we observed a different effect of thermal denaturation on the copying of A-T sequences depending on the method of preparation. Form I was always activated by this treatment but to a lesser extent than total DNA. Form II behaved like synthetic poly d(A-T) , i.e. thermal denaturation had no positive effect, the reaction having been initially maximal. In certain cases, thermal denaturation even diminished the efficiency of such a template. This phenomenon must correspond to certain modifications observed in other respects such as a decrease in the number of single-strand breaks, i.e. in 3'OH ends, during the heating process. These results agree with the structural differences between Form I (native structure) and Form II (incompletely renatured) as pointed out in the preceding article ⁷.

Finally, when we compared a poly d(A-T) enriched DNA obtained by chromatography on methylated albumin, with mixtures of poly d(A-T) and main component containing the same proportion of poly d(A-T), we observed the following : the enriched DNA (Fig. 1e) still behaved like total DNA

Figure 1 : Influence of thermal denaturation on template activity of total crab DNA and its two components. The reaction was performed under the conditions described in "Material and Methods" ; a) b) d) 12.5 µg of template; e) f) g) 10 µg of template, i.e. optima concentrations of template per assay ; c) 2 µg of template because of the small quantities of poly d(A-T) that could be prepared by Davidson's method ; ——— the template was used in native state ; ----- the template was previously heat denatured.

and was sensitive to thermal denaturation for the copying of both A-T and G-C sequences. In contrast, in the f) and g) mixtures composed of poly d(A-T) (Form II) and main component isolated on hydroxylapatite or on methylated albumin, only the incorporation of dGMP was increased by template denaturation. The A-T sequences were copied as in the case of isolated poly d(A-T) whether the template was native or heat denatured. The rate of synthesis was also much higher than that obtained with DNA enriched on MAK and containing the same proportion of poly d(A-T).

The same results were obtained with mixtures containing 50% of synthetic poly d(A-T) plus 50% of main component and to a lesser degree, with mixtures containing 50% of poly d(A-T) (Form I) plus 50% of main component.

Influence of premelting at 70°C :

We heated the material at 70°C to determine the importance of the secondary structure in the template activities of the different DNAs with regard to purified DNA polymerase. This temperature corresponds to the melting point of poly d(A-T) but is clearly lower than that for the main component ¹⁴.

Figure 2 (A-B-C) shows the results for total DNA, for a DNA that had been enriched in poly d(A-T) on a methylated albumin column (but where the poly d(A-T) conserved its native structure), and a poly d(A-T) obtained by Davidson's method (Form I). These templates were studied in the native state, after heating at 70°C followed by gradual cooling, or after heating at 100°C with sudden chilling in ice water. This experiment confirms our first results and the physicochemical results previously published ⁷.

Poly d(A-T) - Form I was isolated in its native form but heating at 70°C led to a melting of the molecule. The G.C base pairs that are distributed along the length of the polynucleotide chain and the non-alternating sequences of A.T base pairs ¹³ are separated and do not renature. This results in a partially renatured molecule whose template activity with the DNA polymerase is maximal. Heating at 100°C followed by

Figure 2 : Influence of a premelting at 70°C on template activity of crab DNA. Template : 10 µg per assay. A - Whole crab DNA - B - poly d(A-T) enriched DNA obtained by chromatography on methylated albumin. C - poly d(A-T) Form I. These samples are a generous gift from Dr. A.M. Michelson. Each preparation, in 0.01 M NaCl, 0.005 M cacodylate pH 7, was used : a) native, b) after heating at 70°C and slow cooling, c) after heating at 100°C and fast cooling in cold water.

sudden chilling did not result in as good a template (Fig. 2C). This shows the effect of the 100°C heating on poly d(A-T) Form II, which had already been heated to 70°C during its isolation, and can also explain the difference observed between enriched DNA and reconstituted mixtures (Fig. 1).

In whole DNA and in DNA enriched in poly d(A-T) on the MAK column, the 70°C heating allows a greater copy of C-rich regions (Fig. 2A and 2B). The percentage of G-C in the regions rendered accessible to the enzyme by such a treatment is much greater than the 2 to 3% of the G.C base pairs of poly d(A-T). This presents a problem that we shall discuss later.

Action of Actinomycin :

Actinomycin binds to the G bases of a double-stranded DNA and at certain concentrations inhibits DNA polymerase by interfering with the separation of the two strands of the template ¹⁵.

It has been demonstrated with E. coli RNA polymerase that under enzyme-limiting conditions, the actinomycin added to a mixture of thymus DNA and syntetic poly d(A-T), blocks the copy of thymus DNA and increases the copy of poly d(A-T) ¹⁶. If the poly d(A-T) and the main component of crab DNA are two independent molecules, the addition of actinomycin to whole DNA must prevent the fixation of the DNA polymerase on the main component rich in G-C and favorise the copy of poly d(A-T). We therefore tried to determine the extent that actinomycin can block or facilitate the copy of crab poly d(A-T) isolated or not from whole DNA. We followed the incorporation of the 4 deoxynucleoside triphosphates in the presence of total DNA, main component or poly d(A-T), native or heat denatured, using optima concentrations of template.

The duplication of isolated poly d(A-T) (Form I or Form II) is only slightly affected by actinomycin as the copy of the G-C sequences contained in this satellite. This confirms the results of Hyman & Davidson ⁴ according to which 40% of the G.C bases of crab poly d(A-T) are not susceptible to actinomycin binding and the results of Bernardi's group showing that 65% of G.C base pairs are in isolated positions ¹³. The same is observed after thermal denaturation (Fig. 3c).

With the main component (43% G.C base pairs) however, actinomycin works in opposition to the action of thermal denaturation and strongly inhibits replication of both G-C and A-T sequences (Fig. 3d). Actinomycin's action may be an inhibition of the strand separation normally required for replication of template ¹⁵.

Figure 3 : Actinomycin effect on template activity of total crab DNA and its two components
 Template : 12,5 µg per assay ; — control ; - - - - plus actinomycin ; same results
 were obtained with heat denatured poly d(A-T) (Form II) ; in its native state, the main
 component is a very poor template for DNA polymerase.

The sequences copied in native total DNA are only slightly sensitive to this antibiotic ; under these conditions, the enzyme must copy the sequences very rich in A-T, but not uniquely poly d(A-T) (dissymmetric incorporation of [^3H]dAMP and [^3H]dTMP). In no case, could we demonstrate a positive effect of actinomycin on the copy of the satellite (Fig. 3a). In contrast, when total DNA was denatured by heat, actinomycin strongly blocked the copy of both G-C and A-T sequences. By intercalating between the partially renatured G-C sequences, actinomycin stabilizes the double-stranded portions of the molecule which therefore cannot be opened by the enzyme (Fig. 3b).

In the same manner, we compared a poly d(A-T) enriched DNA, obtained by chromatography on methylated albumin, to mixtures of poly d(A-T) and main component containing the same proportions of poly d(A-T) (not shown). As seen in the preceding paragraph, the enriched DNA still behaved as total DNA, whereas in reconstituted mixtures, poly d(A-T) (Form I or II) was not sensible to actinomycin action before or after thermal denaturation.

Pretreatment of the template with *Neurospora crassa* nuclease :

The use of this nuclease, specific for the degradation of single polynucleotide chains⁹, allowed us to obtain the following results :

- Total DNA was practically undegraded by this enzyme in its native state. The sequences copied by purified DNA polymerase were therefore, double-stranded. In contrast, if the nuclease of *N. crassa* was allowed to react with heat denatured total DNA, the template capacities of it were almost entirely suppressed (Fig. 4a).

The copy of poly d(A-T) (Form I) was not modified by pretreatment with *Neurospora crassa* nuclease, which confirms its native structure (Fig. 4b, I).

- The pretreatment of poly d(A-T) (Form II) with this enzyme, in contrast led to a large drop in the incorporation of [^3H]dAMP and [^3H]dGMP. The incorporation of dAMP was about 75% of that which was obtained with the same untreated poly d(A-T). The incorporation of dGMP reached only 20% of control values (Fig. 4b, II).

Role of certain nucleotide sequences in the initiation of in vitro duplication :

Kinetic studies of the incorporation of [^3H]dGMP and of [^3H]dAMP in the presence of poly d(A-T) prepared on hydroxylapatite (Form II) showed that the percentage of incorporation of [^3H]dGMP was quite superior to the

Figure 4 : Pretreatment of the template with *Neurospora crassa* nuclease. After preincubation of the template with *Neurospora crassa* nuclease, the polymerization reaction was started by addition of the deoxynucleoside triphosphates and DNA polymerase ; a) whole crab DNA : native (N) or heat denatured (D) ; b) poly d(A-T) separated by density gradient (I) or by chromatography on hydroxylapatite (II). In ordinate the polymerization of dAMP or dGMP expressed in % of control.

theoretical percentage of this base in the template (Table 1). This phenomenon was noted with poly d(A-T) Form I in the first 15 minutes of the reaction ; the percentage of G.C base pairs of the product formed stabilized quickly thereafter to 5%, i.e., to a value very close to the theoretical percentage (Table 1). For total DNA or the main component, the percentage of dGMP incorporated in the presence of these native templates quickly dropped below the theoretical percentage of this base in the template. In contrast, when the templates were previously heat denatured, we observed that the incorporation of dGMP was constant during the reaction and that the product formed was richer in dGMP than the template utilized.

Knowing that the DNA polymerase of regenerating rat liver has a strong affinity for single-stranded poly (dC) sequences ⁶, we wanted to verify whether such sequences could explain the preferential incorporation of dGMP at the beginning of the reaction. For that, we pre-treated

TABLE 1

BASE COMPOSITION OF THE TEMPLATE AND THE PRODUCT SYNTHESIZED

Template : Theoretical % of G-C			Product : % G-C calculated from the incorporation of dGMP and dAMP				
			5 min	10 min	15 min	30 min	60 min
Total DNA :	Native	36,3	45	36	38	33	30
	Denatured	"	-	59	57	56	55
Main component	Native	42,7	35	27	28	27	23
	Denatured	"	59	59	59	57	57
<u>Poly d(A-T) :</u>							
Form I		2-4	17	13	6	5	5
Form I + N.c nuclease			22	10	5	6	5
Form II		2-4	25	21	22	16	16
Form II + N.c nuclease			12	13	12	6	5

poly d(A-T) (Form I and Form II) with Neurospora crassa nuclease. After reaction of the nuclease on poly d(A-T) (II), we observed a large drop in the incorporation of dGMP which approached the theoretical values of the template (Table 1). As we have seen above, the incorporation of dAMP was about 75% of that obtained with the same non-treated poly d(A-T). For both A-T and G-C sequences, we can observe the importance of the single-stranded portions in the initiation of the in vitro duplication (Fig.4b,II).

Poly d(A-T) isolated on a density gradient in its native form (Form I) however, was only slightly attacked by the Neurospora crassa nuclease. In this case, the preferential incorporation of dGMP took place in the first few minutes of the reaction (Table 1).

DISCUSSION

The study of the template activities of crab DNA and of its two components, poly d(A-T) and main component, confirms the properties of the regenerating rat liver DNA polymerase that we previously demonstrated using synthetic polymers :

a) the importance of the single-stranded poly (dC) sequences in the initiation of in vitro duplication,

b) the importance of the secondary structure of the template for the DNA-dependent DNA polymerase of high molecular weight. The A-T alternating sequences are the only ones that can be copied by the enzyme in its double-stranded form. Heating at 70°C, which breaks the hydrogen bonds between the non-alternating A-T sequences and the G.C base pairs of the satellite, increase its template activity.

In addition, our results raise the question concerning the continuity of poly d(A-T) and main component within the intact DNA. Actinomycin when added to whole DNA, under enzyme-limiting conditions, never favored the copy of poly d(A-T) as it should have if the main component and the satellite are two independent molecules. Furthermore, the 70°C heating of whole DNA and DNA enriched in poly d(A-T) on a MAK column permits the copy of regions with a greater G.C percentage than the satellite. Finally, whatever the method of poly d(A-T) preparation, the incorporation of dGTP at the beginning of the reaction is preferential. This latter result cannot be explained by a contamination of poly d(A-T) by main component since this is a very poor template for DNA polymerase without thermal denaturation. These results seem to indicate that the poly d(A-T) can be bound to the main component in intact crab DNA. The richer G-C sequences of the

latter slow the opening and, consequently, the copy of poly d(A-T) to its native state, and are, in contrast, partially "melted" when the A-T rich satellite is denatured at 70°C. Poly d(A-T) would alternate with the main component along the same DNA molecule as has been shown with other highly repetitive sequences ¹⁷.

In support of this hypothesis, we can cite the results that we obtained by another approach. Hybridization in situ (using the highly radioactive copy of poly d(A-T) synthesized in our system in vitro) and different cytochemical techniques, showed that crab poly d(A-T) was uniformly dispersed throughout the nucleus in contrast to certain centromeric satellites ^{18,19}. This problem is now being studied by electron microscopy.

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Abbreviations used : MC, main component ; N.c nuclease, Neurospora crassa nuclease ; MAK, methylated albumin-kieselguhr.